ISOLATION OF HALOPHILIC LACTIC BACTERIA *TETRAGENOCOCCUS HALOPHILUS* FROM VIETNAMESE FISH SAUCE

To Thi Nga, Dang Xuan Tra, Le Thanh Ha*

School of Biotechnology and Food Technology, Hanoi University of Science and Technology, 1 Dai Co Viet, Hai Ba Trung, Ha Noi

*Email: *ha.lethanh@hust.edu.vn*

Received: 28 August 2017; Accepted for publication: 12 October 2017

ABSTRACT

Halophilic lactic bacteria *Tetragenococcus halophilus* were isolated from Thailand fish sauce. They were suggested to play an important role in flavor and aroma development and used as starter culture during fish sauce fermentation.

In this study the isolation of *Tetragenococcus halophilus* from two different factories of fish sauce production in Vietnam was conducted on MRS agar media supplemented with 0.5 % $CaCO_3$ and 5 % NaCl under anaerobic condition. The *Tetragenococcus halophilus* was present in all investigated fish sauce samples from 2 months to 8 months. Six isolates CH2-4, CCH8-3, CH6-1, CH6-2, CH8-1 and V5-1 were selected based on various ability to grow in fish broth supplemented with 25 % NaCl and degrade fish protein to oligopeptide. The 16S rDNA sequence analysis of 6 isolates showed that they were closely related to *Tetragenococcus halophilus* ATCC 33315 and *Tetragenococcus halophilus* MCR10-7-8 and MRC 5-5-2 from study of Thailand scientist with more than 99 % sequence homology, suggesting their potential using as starter culture in fish sauce fermentation.

Keywords: fish sauce, halophilic lactic bacteria, *Tetragenococcus halophilus*, proteolytic activity.

1. INTRODUCTION

Fish sauce is popular seasoning in Southeast Asia including Vietnam. The fish sauce is produced through a natural fermentation process whereas indigenous microorganisms play an important role. The process prolongs for more than 12 months. Many attempts focused on using starter cultures to reduce the fermentation time and improve the flavor and aroma of fish sauce like *Staphylococcus* [1], *Virgribacillus* [2], or mixture of *Bacillus* and *Lactobacillus* [3], *Tetragenococcus* [4]. Among microorganisms used as starter culture, lactic acid bacteria are preferred.

Lactic acid bacteria (LAB) are the most common microorganisms used for fermentation in food industry. LAB are considered GRAS bacteria i.e. totally safe for human consumption. Among halophilic lactic acid bacteria, *Tetragenococcus* are the most common in high-salt

fermented food products. This bacterium has been found in various food products, such as salted anchovies [5], Japanese soya sauce [6], Japanese traditional fermented fish sauce (squid liver sauce) [7]. Recently, they have been isolated from Japanese-fermented puffer fish ovaries [8], Indonesian terasi shrimp paste [9], Cambodian fermented fish [10]. The dominant *Tetragenococcus* in the final stage of fish sauce fermentation when aroma, colour and flavor developed was confirmed by metagenomic method, suggesting their role for flavor and aroma development [11]. By culturing method, Udomsil [12] found that LAB was isolated from nampla fish sauce from the first month to twelfth month ranged from 2.11 to 3.76 log cfu/ml for one factory and from 3.26 to 4.26 log cfu/ml for another. The subsequent identification showed that they all belonged to *Tetragenococcus halophilus* [12]. The use of *Tetragenococcus halophilus* as starter culture for enzyme hydrolyzed fish protein could reduce the fermentation time to 6 months without affecting the quality of the fish sauce product [11].

The study of *Tetragenococcus* from fish sauce in Vietnam is still moderate. Thus the aim of this study was isolation and identification of *Tetragenococcus* from Vietnamese fish sauce, their ability to grow in high-salt environment and their proteolytic activity for further used as starter culture in fish sauce fermentation.

2. MATERIALS AND METHODS

2.1. Materials

Fish sauce samples were collected from Cat Hai factory (Haiphong) in North and Cua Hoi factory (Vinh) in Middle of Viet Nam.

Chemicals for MRS media were of bacteriological grade and were purchased from LAB (UK) and Merck (Germany). NaCl for media was of analytical grade (China). Chemicals for analysis of analytical grade were purchased from Merck (Germany). Kit API 50 CHL was purchased from Biomeriex (France). Fresh fish (*Sardinella aurita*) was purchased in Thanh Hoa, stored in ice and transferred to lab.

2.2. Isolation of halophilic lactic acid bacteria (LAB) from fish sauce fermentation samples

The fish sauce sample (500 mL including mash and liquid) were collected from Cat Hai and Cua Hoi fish sauce factories at 2, 4, 6, 8 months and 3, 5, 7 months of fermentation, respectively. LAB was isolated from samples using MRS agar containing 5 % NaCl supplemented with 0.5 % $CaCO_3$ by spread method. The plates were incubated at 30 °C for 3-5 days under anaerobic condition using an anaerobic chamber. Seventeen isolates with different morphological characteristics were randomly selected. The isolates were purified on the same medium and stored in Glycerol 10 % at -20 °C. Before proteolytic activity test, they were identified by morphological and catalase test.

2.3. Proteolytic activity assay of halophilic LAB

Seventeen selected isolates were tested for proteolytic activity using fish broth containing 25 % NaCl (FB25). Fish broth was prepared by boiling 1 part of fish with 2 parts of distilled water for 20 min. The mixture was then filtered through cheesecloth and supernatant was collected. Media was autoclaved at 121 °C for 15 min [4]. The isolates were cultured in MRS broth containing 10 % NaCl for 4 days, then seed culture subsequently was inoculated in FB25

at ratio 2 % (v/v) and incubated at 30 °C for 7 days under anaerobic condition using an anaerobic chamber. To ensure the similar cell number of different isolates in FB25, the seed culture was centrifuged, removed supernatant, and the cells were resuspended in certain volume of sterile 0.9 % NaCl to obtain the OD600 of 1.0 (approximate 10^6 CFU/ml). The pH of FB25 after 7 days incubation was measured using a pH meter. LAB counts were determined by spread-plate method using MRS agar containing 5 % NaCl and 0.5 % $CaCO_3$ at 30 °C for 3-5 days under anaerobic condition.

Cell cultures in fish broth after 7 days incubation were centrifuged at 10000×g for 10 min at 4 °C, and supernatants were collected and used for determination of protein and oligopeptide contents.

Remaining protein content in fish broth and cultured supernatant was determined by Bradford protein assay using bovine serum albumin (BSA) as a standard [8]. Trichloroacetic acid (TCA)-soluble oligopeptides content in fish broth and cultured supernatant was determined by adding 20 % TCA to supernatant at a ratio 1:1 leaving at 4 °C overnight and subsequently centrifuging at 10000×g for 10 min at 4 °C, after that oligopeptides content was determined by Lowry method using tyrosine as a standard [8]. The residual protein and oligopeptide content relative to the respective original values (FB without inoculating LAB) were calculated and expressed in percentage. Six isolates were selected based on ability to grow on FB25 and proteolytic activity.

3.3. Identification of selected LAB isolates

The cell shape and cell arrangement were observed by Gram staining. Catalase test was conducted according to Bergey's Manual of Determinative Bacteriology. Biochemical test was caried out using Kit API 50 CH/CHL for LAB (Biomerieux) according to manufacturer's guide. In addition, the isolates were further identified by 16S rRNA gene sequence analysis. For that genomic DNA was extracted and used for PCR using primer (5' AGAGTTTGATCMTGGCTCAG 3' and 5' TACGGYTACCTTGTTACGACTT 3'). The PCR products were purified using kit PureLink™ – DNA Purification (Invitrogen) and sent to First BASE (Malaysia) for sequencing. Phylogenetic tree was constructed by the Maximum Parsimony method with software MEGA.

3. RESULTS AND DISCUSSION

3.1. Isolation of halophilic lactic acid bacteria (LAB) from fish sauce fermentation samples

The lactic acid bacteria (LAB) in the fish sauce samples fermented for 2, 4, 6, 8 months from Cat Hai and 3, 5 and 7 months from Cua Hoi factories were isolated according to the method described in Materials and Methods. The samples of 8 months from Cat Hai was by fermented by two methods (Cat Hai and Phu Quoc method).

LAB presented in all fish sauce samples from Cat Hai and Cua Hoi factories, ranged from 3.3 to 7.6 log CFU/ml. LAB count from Cat Hai were generally higher than those from Cua Hoi factory. The LAB count from our sample was higher than from Udomsil study [12], especially those samples fermented for 2-3 months. This could be due to variation of raw material and fermentation methods among factories. Kilinc *et al*. reported that LAB counts during 1 to 57 days of fish sauce fermentation in 6 samples were various but followed the same tendency. The number of LAB counts in all six fish sauce samples increased from day 1 (ranged 3.88–4.34 log

CFU/ml) to day 8 (ranged 6.62 - 7.92 log CFU/ml). After reaching maximum, the LAB counts decreased until the day 57 to initial or a slightly higher number at day 1 (3.86–5.49 log CFU/ml) [13]. Ijong and Ohta (1996) found a variety of LAB during bakasang (Indonesian fish sauce) fermentation, namely *Lactobacillus* and *Streptococcus* at counts 4-6 log CFU/ml [14]. LAB were found throughout 12 months of fermentation in Udomsil's study at counts 2.11 to 4.26 log CFU/ml but the LAB counts did not follow decrease tendency as those in our study. The LAB counts in our study was higher than in previous reported study. The different method of fermentation could explain this variation. Thus the results of our study confirmed the present of LAB in Vietnamese fish sauce mash at quite high number and decreased during fermentation.

Table 1. Lactic acid bacteria counts (log CFU/ml) of samples collected from two different factories.

Fish sauce production factories	Fermentation time (month)	Total viable counts (log CFU/ml)
Cat Hai	2	7.60
	4	5.48
	6	5.70
	8 (Cat Hai)	4.56
	8 (Phu Quoc)	4.11
Cua Hoi	3	6.23
	5	4.30
	7	3.30

3.2. Growth and proteolytic activity of isolates

A total of 17 isolates from fish sauce samples was randomly selected. All 17 isolates were tested for cell morphology and catalase activity. The cell shape and cell arrangement were observed by Gram staining. Catalase test was conducted according to Bergey's Manual of Determinative Bacteriology.

They all were Gram positive cocci with cell arrangement as pairs/tetrads form and catalase positive. The gram staining of 9 selected isolates were presented on Figure 1. Colonies on MRS agar were 1-2 mm, white, circular, low convex, and smooth similar to those in Udomsil's study (Data were not shown). The 17 isolates were tested for ability to grow in high salt environment and proteolytic activity. Fish broth containing 25 % NaCl (FB25) was used for this purpose. Fish broth contained mainly fish extract, the main raw material used for fish sauce fermentation. The chemical composition of fish broth would be comparable to that of fish sauce fermentation [12]. Since the selected LAB were intended to be used as a starter culture for fish sauce fermentation, LAB able to grow in FB25 were likely to survive during fish sauce fermentation. Almost tested LAB showed an increase of cell count approximately 1-2 log CFU/ml within 7 days except CCH8-3, CH2-3 and V7-2 (Table 2). It could be seen that CH2-4, CH4-2, V3-1 and V3-2 showed good growth in high salt environment; the cell counts reached highest 2.17 log cfu/ml by isolate CH2-4 (Table 2) which slightly lower than that of Udomil's study 2.63 [12].

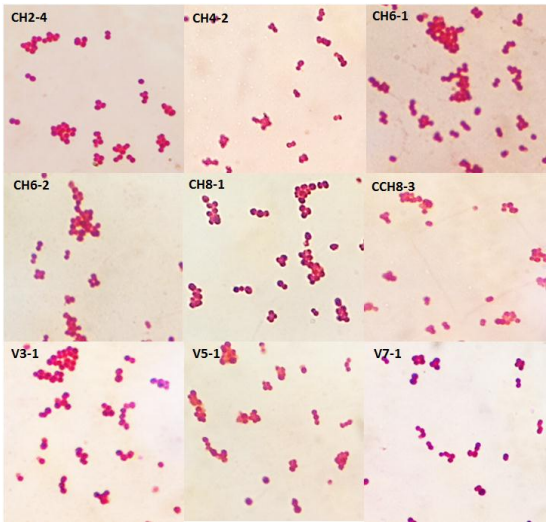

Figure 1. Gram stain and cell arrangement of selected LAB isolated from Cat Hai fish sauce 2nd month (CH2-4), 4 th month (CH4-2), 6th month (CH6-1, CH6-2), 8th month (CH8-1) fermented by Cat Hai method, 8th month (CCH8-3) fermented by Phu Quoc method and 3, 5 and 7th month from Vinh fish sauce (V3-1,V5-1, V7-1).

Table 2. Changes of bacterial growth, protein, oligopeptide content and pH value of fish broth containing 25 % NaCl inoculated with 17 isolates.

Factory	Fermentation period (month)	Bacterial isolate code	Δ Growth (Log CFU/ml)	Δ Protein content (%)	Δ Oligopeptide (%)	pH
Cat Hai	2	CH2-1	1.45	-15.68	4.42	6.28
		CH2-2	1.58	-17.63	2.64	6.16
		CH2-3	0.59	-17.43	6.41	6.14
		CH2-4	2.17	-10.76	7.56	6.12
	4	CH4-2	1.70	-19.37	5.64	5.89
	6	CH6-1	1.37	-4.51	7.88	6.22
		CH6-2	1.37	-13.02	7.17	6.1
	8	CH8-1	1.35	-11.69	7.57	6.15
		CH8-2	1.05	-18.76	9.64	5.95
	8	CCH8-1	1.45	-11.17	11.41	6.04
		CCH8-2	1.51	-21.53	6.75	6.27
		CCH8-3	-0.26	-11.38	3.32	5.89
Cua Hoi	3	V3-1	1.77	-5.33	6.27	6.18
		V3-2	1.68	-14.25	7.76	6.11
		V3-3	1.36	-22.14	5.99	6.13
	5	V5-1	1.39	-7.59	6.56	6.06
	7	V7-2	0.86	-3.90	8.64	6.21

The pH of FB25 after 7 days decreased slightly from initial pH 6.3 to pH in range from 5.89 to 6.28, which were lowest by isolates CH4-2 and CCH8-3. The decrease of pH might be due to lactic acid production by LAB. The decrease of protein content indicated the ability of isolates to hydrolyze fish protein to oligopeptide/aminoacid for growth or for converting to other compound. Some isolates utilized much fish protein up to 22 % but some utilized only 3.9 % FB protein. Proteinases produced by LAB could hydrolyze fish protein at high NaCl concentration revealing that isolated LAB might produce salt-stable proteinases. Some isolates of Udomsil's study could utilize up to 36 % fish protein, which was more than 1.5 times higher than ours [12]. All isolates resulted in an increase of oligopeptide content, indicating their proteolytic activity (Table 2). Proteolysis is essential for liberating amino acids for flavor development [15]. The slight or strong increase of oligopeptide might be not related to high proteolytic activity since oligopeptides were utilized for bacterial growth or for flavor development. Whereas all our isolates resulted on increase of oligopeptide, some isolates from Udomsil's study resulted in decrease of oligopeptide.

In order to assess the diversity of lactic acid bacteria presented in fish sauce samples, isolates with different characteristics such as isolates with high ability to growth in FB25 like CH2-4, with lowest pH after 7 days like CCH8-3, utilized low FB protein content like CH6-1 were selected for further study. Besides, the isolates with various fermentation time and from different factories, with reasonable ability to growth in FB25, average amount of oligopeptide and remaining protein content CH8-1, CH6-2 , V5-1 were also selected.

3.3. Identification of selected LAB isolates

3.3.1. Identification of isolates by 16S rRNA sequence analysis

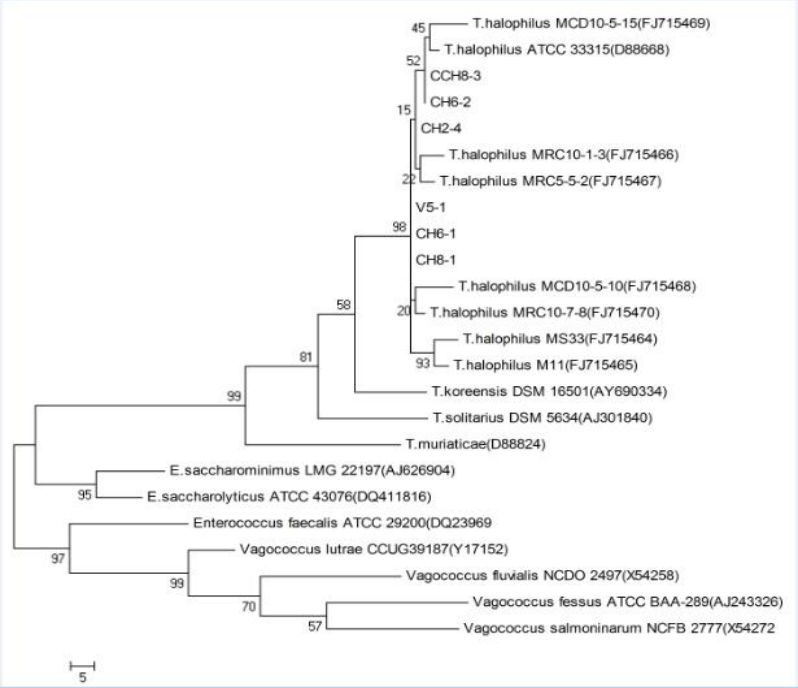

Figure 2. Phylogenetic tree of lactic acid bacteria isolated from fish sauce fermentation, based on 16S rRNA gene sequence data. Bar indicates 5 substitutions per nucleotide position.

When phylogenetic tree analysis was performed, all six isolates fell in the same cluster of *T. halophilus* ATCC 33315 and *T. halophilus* MCR10-7-8 and MRC 5-5-2 from Udomsil's study (NCBI code from FJ15470 and FJ154670) (Figure 2) with 99 % sequence homology. Therefore, six isolates of LAB from fish sauce fermentation in this study were identified as *T. halophilus.*

The positive effect of Udomsil's isolates for using as starter culture in fish sauce fermentation [4] promised the potential of using isolates from this study. In his study, Thongsanit *et al*. reported the presence of both *T. halophilus* and *T. muriaticus* [16]. In our study, only *T. halophilus* was found that was similar to result of Udomil's study. *T. halophilus* might be the prevalent LAB in fish sauce fermentation. From the *Figure* 2, it can be seen that 6 isolates could divide in 3 group based on 16S rRNA sequence analysis. Group 1 included CH6-1, CH8-1 and V5-1, group 2 included CCH8-3 and CH6-2, CH2-4 belonged to group 3. Therefore the CH6-2, CH2-4, CH8-1 and CCH8-3 were selected for further biochemical test.

3.3.2. Biochemical characteristics of selected bacterial isolates

Biochemical test was caried out using Kit API 50 CH/CHL for LAB (Biomerieux). Results indicated that isolates CH2-4 and CH8-1 produced acid from L-arabinose, D-galactose, D-glucose, D-fructose, inositol, Methyl-β D-glucopyranoside, D-cellobiose and D-melibiose. Isolate CCH8-3 fermented only D-fructose and D-melibiose. Isolate CH6-1 fermented only Methyl-β D-glucopyranoside. Similar to *Tetragenococcus halophilus* ATTC 33215, isolate CH2-4 and CH8-1 produced acid from L-arabinose, whereas none of isolates from Udomsil's study did. The results revealed the diversity of *Tetragenococcus halophilus.*

4. CONCLUSIONS

The halophilic lactic acid bacteria was found in all fish sauce sample throughout second to eighth month of fermentation with decreasing tendency of cell number. *Tetragenococcus halophilus* seemed prevalent in halophilic LAB isolated from fish sauce and could withstand until eight month in high salt environment. All selected isolates possessed proteolytic activity functioned at high salt environment. Ribosomal 16S RNA sequence analysis showed more than 99 % sequence homology of six selected *Tetragenococcus halophilus* with *T. halophilus* MCR10-7-8 and MRC 5-5-2 from Thailan scientists study, which have been showed positive using as starter culture for fish sauce fermentation. The results suggested potential of six isolated strains from this study. Their volatile compounds profile and peptidase activity need to be further investigated to confirm their potential.

Acknowledgments. The authors would like to thank Ministry of Education and Training for providing fund for project BKA-2017-15A to pursue this research.

1. REFERENCES

2. Udomsil N., Rodtong S., Tanasupawat S., and Yongsawatdigul J. - Improvement of Fish Sauce Quality by Strain CMC5-3-1: A Novel Species of *Staphylococcus* sp, Journal of Food Science **80** (9) (2015) 2015-2022.
3. Yongsawatdigul J., Rodtong S., and Raksakulthai N. - Acceleration of Thai Fish Sauce Fermentation Using Proteinases and Bacterial Starter Cultures, Journal of Food Science **72** (9) (2007) 382-390.

4. Nguyễn T. V. A., Bùi T. T. H., Nguyễn Đ. T., and Nguyễn T. H. - Tuyển chọn vi khuẩn lactic và *Bacillus* có khả năng chịu muối cao ứng dụng cho quá trình sinh hương trong sản xuất nước mắm, Hội nghị KHCN sinh học toàn quốc 2013 **2** (2013) 54-59.
5. Udomsil N., Rodtong S., Choi Y. J., Hua Y., and Yongsawatdigul J. - Use of *Tetragenococcus halophilus* as a Starter Culture for Flavor Improvement in Fish Sauce Fermentation, Journal of Agricultural and Food Chemistry **59** (15) (2011) 8401-8408.
6. Villar M., de Ruiz Holgado A. P., Sanchez J. J., Trucco R. E., and Oliver G. - Isolation and characterization of *Pediococcus halophilus* from salted anchovies (Engraulis anchoita), Applied and Environmental Microbiology **49** (3) (1985) 664-666.
7. Sakaguchi K. - Studies on the Activities of Bacteria in Soy Sauce Brewing, Bulletin of the Agricultural Chemical Society of Japan **22** (6) (1958) 345-362.
8. Satomi M., Kimura B., Mizoi M., Sato T., and Fujii T. - Tetragenococcus muriaticus sp. nov., a New Moderately Halophilic Lactic Acid Bacterium Isolated from Fermented Squid Liver Sauce, International Journal of Systematic and Evolutionary Microbiology **47** (3) (1997) 832-836.
9. Kobayashi T., Kimura B., and Fujii T. - Differentiation of *Tetragenococcus* populations occurring in products and manufacturing processes of puffer fish ovaries fermented with rice-bran, International journal of food microbiology **56** (2-3)(2000) 211-218.
10. Kobayashi T., Kajiwara M., Wahyuni M., Kitakado T., Hamada-Sato N., Imada C., and Watanabe E. - Isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: A traditional fermented seafood product in Indonesia, The Journal of General and Applied Microbiology **49** (5) (2003) 279-286.
11. Chuon M. R., Shiomoto M., Koyanagi T., Sasaki T., Michihata T., Chan S., Mao S., and Enomoto T. - Microbial and chemical properties of Cambodian traditional fermented fish products, J Sci Food Agric **94** (6) (2014) 1124-31.
12. 11. Lee S. H., Jung J. Y., and Jeon C. O. - Bacterial community dynamics and metabolite changes in myeolchi-aekjeot, a Korean traditional fermented fish sauce, during fermentation, Int J Food Microbiol **203** (2015) 15-22.
13. Udomsil N., Rodtong S., Tanasupawat S., and Yongsawatdigul J. - Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds, International Journal of Food Microbiology **141** (3)(2010) 186-194.
14. Kilinc B., Cakli S., Tolasa S., and Dincer T. - Chemical, microbiological and sensory changes associated with fish sauce processing, European Food Research and Technology **222** (5) (2006) 604-613.
15. Ijong F. G. and Ohta Y. - Physicochemical and Microbiological Changes Associated with Bakasang Processing - A Traditional Indonesian Fermented Fish Sauce, Journal of the Science of Food and Agriculture **71** (1) (1996) 69-74.
16. Smit B. A., Engels W. J. M., and Smit G. - Branched chain aldehydes: production and breakdown pathways and relevance for flavour in foods, Applied Microbiology and Biotechnology **81** (6) (2009) 987-999.
17. Thongsanit J., Tanasupawat S., Keeratipibul S., and Jatikavanich S. - Characterization and Identification of *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* Strains from Fish Sauce (Nam-pla), Japanese journal of Lactic acid Bacteria **13** (1) (2002) 46-52.

TÓM TẮT

PHÂN LẬP CHỦNG *TETRAGENOCOCCUS HALOPHILUS* CHỊU MẶN TỪ NƯỚC MẮM VIỆT NAM

Tô Thị Nga, Đặng Xuân Trà, Lê Thanh Hà*

Viện Công nghệ sinh học và Công nghệ thực phẩm, Trường Đại học Bách khoa Hà Nội

*Email: *ha.lethanh@hust.edu.vn*

Vi khuẩn lactic chịu mặn *Tetragenococcus* đã được phân lập từ nước mắm Thái Lan và được chứng minh đóng vai trò quan trọng cho quá trình tạo hương và vị cho nước mắm, có thể sử dụng như chủng khởi động cho lên men nước mắm.

Trong nghiên cứu này, quá trình phân lập *Tetragenococcus halophilus* từ hai nhà máy nước mắm Việt nam đã được thực hiện trên môi trường MRS bổ sung 0,5 % CaCO3 và 5 % NaCl trong điều kiện yếm khí. Kết quả cho thấy *Tetragenococcus* xuất hiện trong tất cả các mẫu nước mắm từ tháng 2 đến tháng 8. Sáu chủng CH2-4, CCH8-3, CH6-1, CH6-2, CH8-1 và V5-1 đã được lựa chọn dựa trên khả năng sinh trưởng của chúng trên môi trường dịch cá bổ sung 25 % NaCl và thủy phân protein cá thành oligopeptide. Phân tích trình tự 16 rRNA của 6 chủng cho thấy chúng có quan hệ gần với *Tetragenococcus halophilus* ATCC 33315 cũng như *Tetragenococcus halophilus* MCR10-7-8 và MRC 5-5-2 trong nghiên cứu của các nhà khoa học Thái Lan với hơn 99 % trình tự tương đồng. Kết quả nghiên cứu gợi ý tiềm năng sử dụng 6 chủng tuyển chọn làm chủng khởi động cho quá trình lên men nước mắm.

Từ khóa: nước mắm, vi khuẩn lactic chịu mặn, *Tetragenococcus halophilus*, hoạt tính protease.